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Macrophages are comprised of resident brain microglia not infiltrating peripheral monocytes acutely after neonatal stroke

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Abstract

Macrophages can be both beneficial and detrimental after CNS injury. We previously showed rapid accumulation of macrophages in injured immature brain acutely after ischemia-reperfusion. To determine whether these macrophages are microglia or invading monocytes, we subjected post-natal day 7 (P7) rats to transient 3 h middle cerebral artery (MCA) occlusion and used flow cytometry at 24 and 48 h post-reperfusion to distinguish invading monocytes (CD45high/CD11b+) from microglia (CD45low/medium/CD11b+). Inflammatory cytokines and chemokines were determined in plasma, injured and contralateral tissue 1–24 h post-reperfusion using ELISA-based cytokine multiplex assays. At 24 h, the number of CD45+/CD11b+ cells increased 3-fold in injured compared to uninjured brain tissue and CD45 expression shifted from low to medium with less than 10% of the population expressing CD45high. MCA occlusion induced rapid and transient asynchronous increases in the pro-inflammatory cytokine IL-β and chemokines cytokine-induced neutrophil chemoattractant protein 1 (CINC-1) and monocyte-chemoattractant protein 1 (MCP-1), first in systemic circulation and then in injured brain. Double immunofluorescence with cell-type specific markers showed that multiple cell types in the injured brain produce MCP-1. Our findings show that despite profound increases in MCP-1 in injured regions, monocyte infiltration is low and the majority of macrophages in acutely injured regions are microglia.

Keywords

cytokines; flow cytometry; inflammation; macrophage; microglia; neonatal stroke

Inflammation is a significant contributing factor to neurodegenerative diseases. Depending on injury setting, the relative involvement of systemic and local inflammation, and communication between the two compartments can vary (Carson and Sutcliffe 1999; Baggiolini 2001; Perry 2004). It is well known that stroke in adult triggers a robust inflammatory reaction, largely involving an influx of peripheral leukocytes into the brain parenchyma (for reviews, see Feuerstein *et al.* 1997; Dirnagl *et al.* 1999; Han and Yenari 2003) and disruption of the blood–brain barrier (BBB) (Gidday *et al.* 2005). In the adult, neutrophils are typically the first leukocyte type to infiltrate (Barone *et al.* 1991; Garcia *et al.* 1994; Matsuo *et al.* 1995) and are followed by macrophages and lymphocytes (Barone *et al.* 1991; Garcia *et al.* 1994). The damaging role of infiltrating neutrophils in ischemia was shown by studies in which neutrophil

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depletion (Garcia *et al.* 1994) or administration of anti-adhesion molecules (Kishimoto and Rothlein 1994; Fassbender *et al.* 1999) reversed the reduced local tissue perfusion, BBB disruption, and the release of free radicals, proteinases, and other cytotoxins seen after stroke. The key role of macrophages in cerebral ischemic injury has also been firmly established (Feuerstein *et al.* 1997; Dirnagl *et al.* 1999; Han and Yenari 2003). Macrophage populations in the injured brain, however, are diverse (Carson *et al.* 1998; Dalmau *et al.* 2003) and may consist of infiltrating peripheral monocytes, pericytes, and activated parenchymal microglia. The relative contribution of resident and invading cells may vary depending on injury severity in adult, as is evident from the different dynamics of macrophage accumulation (Morioka *et al.* 1993; Davies *et al.* 1998) and increased expression of the common leukocyte antigen CD45 and α2 integrin CD11b (Campanella *et al.* 2002; Stevens *et al.* 2002) in the tissue after ischemia of different severity. Activated microglial cells are believed to play a significant role in neuro-inflammation after cerebral ischemia (Liu *et al.* 2001; Rosenzweig *et al.* 2004). Distinguishing monocytes from activated microglial cells based on differential antigen expression levels is difficult using conventional immunohistochemical techniques, but can be accomplished using flow cytometry. An understanding of the relative contribution of cells of the monocytic lineage in injury is important for directing the search for novel therapeutic targets.

Compared to the adult, brain susceptibility and the mechanisms of ischemia-related injury are critically affected by immaturity during post-natal brain development (reviewed in Vannucci and Hagberg 2004; Vexler and Ferriero 2006) but less is known regarding the role of the inflammatory component of injury in the neonate. In postnatal day 7 (P7)-P9 rodents, an age when the rat brain is developmentally equivalent to the term human infant brain, up-regulation of structural proteins specific for cells of the monocyte lineage, activated microglia/macrophages, and products of these cells, including cytokines, chemokines and matrix metalloproteinases, have been described following hypoxia-ischemia (McRae *et al.* 1995; Ivacko *et al.*1996; Bona *et al.* 1999; Xu *et al.* 2001; Cowell *et al.* 2002; Hedtjarn *et al.* 2004). Different than in adult status of microglial differentiation (Santambrogio *et al.* 2001) and activation (Carson *et al.* 1998) in the early post-natal period, as well as differences in leukocyte trafficking during normal post-natal development (Lorant *et al.* 1999) and injurious conditions (Anthony *et al.* 1997, 1998 Hudome *et al.* 1997) may substantially affect the rapid accumulation of activated microglia/macrophages in injured tissue that we observed following focal transient middle cerebral artery (MCA) occlusion in P7 rats (Derugin *et al.* 2000; Fox *et al.* 2005; Dingman *et al.* 2006).

It is an entirely unanswered question whether macrophages arise from activation of resident microglia or infiltration of peripheral monocytes in neonatal brain post-ischemia. We used a model of transient MCA occlusion in P7 rats in conjunction with flow cytometry of peripheral and brain leukocytes to determine the relative roles of resident microglia and blood-born macrophages in neonatal stroke. ELISA-based multiplex assays of cytokine and chemokine levels in blood and brain were used to investigate whether inflammatory molecules, including chemoattractants for monocytes, are produced in the brain following neonatal stroke. We found that within the first 24 h after ischemia–reperfusion in the neonate, microglia rather than invading monocytes comprise the macrophage population in injured brain and that production of leukocyte chemoattractants is increased in injured tissue.

Materials and methods

Animal preparation

All experimental procedures were performed in accordance with NIH guidelines for humane handling of animals with prior approval from the Committee of Animal Research at UCSF. Female Sprague Dawley rats with 5–6-day-old-litters were obtained from Simonson Laboratories (Gillroy, CA), given food and water ad libitum and housed in a temperature/light

controlled animal care facility. P7 rats were subjected to 3 h MCA occlusion, as originally reported (Derugin *et al.* 1998) with modifications (Derugin *et al.* 2005). All pups were screened for evidence of injury using diffusion-weighted (DW) MRI 2 h after MCA occlusion and animals with hyperintensity on DW-MRI were selected for experiments (Derugin *et al.* 2000; Manabat *et al.* 2003). At 3 h the suture blocking the MCA was removed, allowing reperfusion, and the pups were returned to the dam. Animals were killed 0–48 h after reperfusion.

Isolation of peripheral leukocytes and brain cells

Trunk blood was collected into tubes containing 200 units Heparin (Elkins-Sinn Inc., Cherry Hill, NJ, USA). Following lysis of RBC with NH_4Cl PharM Lyse buffer (BD Pharmigen, rt × 15 min), leukocytes were collected by centrifugation (250 *g* × 5 min), washed in Hanks Balanced Salt Solution (HBSS) containing 1% BSA (flow buffer) and resuspended in 100 μL buffer and placed on ice.

Injured brain tissue, identified based on abnormalities on DW-MRI during occlusion (Derugin *et al.* 2000; Fox *et al.* 2005) and anatomically matching tissue from contralateral hemispheres were isolated according to published protocols for adult brain (Carson *et al.* 1998; Campanella *et al.* 2002; Stevens *et al.* 2002) with modifications. All steps were performed at 4°C unless indicated. Animals were perfused intracardially with sterile phosphate-buffered saline (PBS) and euthanized by decapitation. Tissue was placed directly into microfuge tubes containing iced RPMI-1640/25 mM Hepes pre-equilibrated with 5% CO_2, homogenized by trituration, and Blendzyme 1 (Roche Applied Sciences, Indianapolis, IN, USA), an endotoxin-free blend of collagenase Types 1 and 2 and the neutral protease Dispase, was added to a final concentration of 45 μg/mL. Gentle isolation with low concentration of Dispase used in our protocol is not associated with degradation of surface markers which was reported using a 100 fold higher Dispase concentration (Ford *et al.* 1996). Tissue homogenates were incubated (30° C × 20 min) with gentle, discontinuous shaking, placed on ice, and enzyme activity was quenched with Fetal Calf Serum (FCS) (10% final concentration). Large debris was removed by filtration over 70 μm filters and cell suspensions were washed with flow buffer. In pilot experiments, CD45/CD11b expression was compared in samples from the same animals obtained with or without use of Percoll gradient purification in which cells were overlayed onto 1.02/1.10 g/mL Percoll gradients (400 *g* × 15 min without brake).

Flow cytometry

All steps were performed on ice or at 4°C unless indicated. For both blood and brain samples, cell surface FcR were blocked (gentle rocking × 15 min) with anti-FcgII R antibody followed by double-labeling of cell surface markers with FITC- and PE-conjugated specific and isotype control antibodies. Unlabeled FcgII R antibody (Fc Block), PE- or FITC-conjugated mouse anti-rat CD45, FITC-CD11b, PE-RP-1 and isotype matched controls were from BD Biosciences (San Diego, CA, USA), PE-CD163 and isotype matched control were from Serotec (Oxford, UK). In each experiment, data from equal numbers of events (10 000–100 000) from control and injured animals (blood) or contralateral and injured brain hemispheres were collected on a FACSCalibur flow cytometer running CellQuest Pro software (BD Biosciences) with the following settings for blood, minor adjustments made as needed: Forward Scotter (FSC) V = E^00, gain = 1.2; Side Scatter (SSC) V = 460, gain = 1.05; thresholding on FSC of 52; compensation FL1 = 1–2%, FL2 = 15–17%. For tissue, modifications to the settings were FSC gain = 1.04; secondary thresholding on SSC of 53; compensation FL2 = 15–25%. Results were analyzed with FlowJo software (Tree Star Inc., Ashland, OR, USA) and are presented for CD45-gated populations. For leukocytes, SSC of 150 was used as the threshold for high versus low SSC populations, for tissue SSC of 250 was used since a clear distinction within the cell populations was detected at these values. Using isotype labeled cells in each

experiment, a gate was drawn containing at least 95% of isotype labeled cells; the population of cells with staining intensity to the right of this gate was classified as CD45 positive (containing < 5% isotype labeled cells). Assignment of CD45 subpopulations into CD45low, med and high populations was based on the boundaries of five percent contour maps (CD45 vs. SSC plots for leukocytes, CD45 vs. FSC plots in control samples) generated by FlowJo software. The number of experiments performed on samples from individual or pooled animals is indicated in figure legends.

Immunocytochemistry

Brains were flash-frozen, stored at − 80°C, and cut coronally (12 μm) on a cryostat and placed on Superfrost Plus glass slides (Fisher Scientific Publishing, Pittsburg, PA, USA). Slides were thawed overnight, fixed in − 20°C acetone × 10 min, washed with PBS and blocked for 1 h in 10% FCS/0.1% Tx-100/.01% NaN_3, as described (Banisadr *et al.* 2005). Sections were incubated with goat anti-rat MCP-1 (1 : 50, Santa Cruz, CA, USA) together with either mouse anti-GFAP (1 : 200, MP Biomedicals, Aurora, OH, USA) or anti-NeuN (1 : 100, Chemicon, Temecula, CA, USA) antibodies in 5% FCS/0.1% Tx-100/.01% NaN_3 overnight at 4°C. Sections were washed with PBS, incubated for 1 h with Cy3-conjugated anti-goat and either Cy2-conjugated anti-mouse or Alexa 488-conjugated isolectin GS-B4 (1 : 100 in 5% FCS/ 0.1% Tx-100/.01% NaN_3), stained with DAPI to label nuclei, washed and coverglassed using Polymount. Images were collected using a Hamamatsu camera and Openlab Software (Improvision, Lexington, MA, USA). Post-acquisition processing of images was performed using identical settings in Adobe Photoshop.

Measurements of cytokine and chemokine levels in plasma and the brain

Concentrations of 14 cytokines, including interleukin-4 (IL-4), IL-1β, IL-1α, IL-6, IL-10, IL-12, IL-18, IL-5, IL-2, interferon gamma (IFNγ), tumor necrosis factor α (TNFα), cytokine induced chemoattractant protein-1 (CINC1/GRO/KC), monocyte chemoattractant protein-1 (MCP-1) and GM-CSF were simultaneously quantified in plasma or brain tissue samples using an ELISA-based bead multiplex assay (Luminex Corp., Austin, TX, USA) as previously reported (Fox *et al.* 2005). Briefly, injured brain tissue and anatomically matching tissue from contralateral hemispheres as well as tissue from control and sham-operated rat pups was homogenized in a buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM PMSF, 0.05% Tween-20, and a cocktail of protease inhibitors (Roche), protein concentration was measured using the BCA assay (Pierce, Rockford, IL, USA). Cytokine and chemokine concentrations were determined using a rat cytokine kit (LINCO Research, Inc., Saint Charles, MO, USA) based on the manufacturer's recommendations. Fluorescent signals originating from cytokine-antibody binding followed by detection with streptavidin-phycoerythrin conjugates were measured with a Luminex^{100} reader. In each experiment, concentrations for each cytokine in the multiplex assay were calculated from calibration curves using individual recombinant proteins as standards dilued in lysis buffer (for tissue) or matrix (for plasma samples). Plasma samples were diluted (1 : 4) in matrix. StatLIA® software (Brendan Scientific Corp., Calrsbad, CA, USA) with a 5-parameter logistic curve-fitting method was used for data reduction, and tissue sample concentrations were normalized to the amount of protein in each sample. Concentrations of several cytokines, including IL-4, IL-1α, IL-10, IL-12, IL-5, IL-2, TNFα, IFNγ and GM-CSF were either consistently below or at the threshold of detection in more than 50% of the samples and therefore not shown.

Statistical Analysis

In flow cytometry experiments cell numbers were analyzed using a two-tailed student's t-test (unpaired for blood, paired for tissue). Cytokine concentrations were analyzed by ANOVA with post-hoc testing (Fisher). Differences were considered significant at $p < 0.05$.

Results

Ischemia-reperfusion triggers activation of peripheral leukocytes

To determine the peripheral leukocyte response to transient MCA occlusion, RBC-depleted leukocytes were analyzed by flow cytometry. In contour plots of CD45 intensity versus SSC, three distinct populations of cells were easily distinguishable in leukocytes from both control and injured animals (Figs 1a and b). Quantitative analysis showed a trend (ns) for greater numbers of CD45+ cells in blood collected from injured animals 24 h post-reperfusion (43 ± 10% events, $n = 8$) compared to uninjured (34 ± 10% events, $n = 8$) animals. Although no rightward curve shift in population histograms of CD45 intensity (Fig. 1c) was detected in leukocytes of injured animals, a medium intensity peak (middle horizontal bar) that appeared as a left shoulder in histograms of leukocytes from control animals clearly increased in magnitude and width with injury. Further analysis was restricted to CD45 gated cells, as these cells represent the total leukocyte population in the sample.

To determine whether ischemia-reperfusion induces expression of the adhesion receptor for ICAM (CD11b/integrin αM) on leukocytes, a receptor needed for leukocyte–endothelium interaction, we colabeled cells with CD45 and CD11b. One major (FI < 10^2, CD11b low) and one minor (FI < 10^3, CD11b high) peak was detected in cells from uninjured animals (Fig. 1d). With injury, the number of cells expressing CD11b increased in both populations, particularly in the population with high signal intensity (at 10^3). In this population, CD11b expression increased significantly from 29 ± 4.8% to 39 ± 6.4% of CD45+ cells (Fig. 1d, $p = 0.05$, $n = 4$). CD45 Vs. SSC plots indicated that the population of CD45+ cells with high SSC (higher granularity) increased with injury, from 24 ± 5.3% to 40 ± 8.4% ($p = 0.003$, $n = 6$). In attempt to determine whether the observed increased SSC represented granulocytes, colabeling experiments were done with CD45 and RP-1, an antibody that recognizes granulocytes but not lymphocytes and monocytes with low SSC. Although there was considerable overlap in intensities between isotype control and specific RP-1 antibodies on leukocytes from control and injured animals, a shoulder in peak RP-1 intensity (< 10^2) was detected in RP-1 stained cells only from injured animals (Fig. 1e), indicating an increase in RP-1 expression after injury. This increased granularity (SSC) and RP-1 expression suggests activation of peripheral neutrophils. Importantly, alterations in CD45 expression and substantial increases in CD11b expression suggest that focal ischemia-reperfusion injury primes leukocytes to be competent to adhere to the endothelium and transmigrate through the microvasculature within 24 h after injury.

Activated microglia, not invading monocytes, are the source of brain macrophages 24 h post-reperfusion

Using immunocytochemical methods we have previously shown increased CD68/ED1 expression in the brain 24 h after neonatal focal stroke (Derugin *et al.* 2000; Fox *et al.* 2005; Dingman *et al.* 2006). To determine the effect of transient MCA occlusion on the pattern of CD45 expression in the brain, flow cytometry was used on cells from contralateral and injured tissue. In pilot experiments with control animals, we found the purification of dissociated cells by Percoll gradient centrifugation, a common component of cell isolation protocols in adult brain used to remove myelin and other debris, to be unnecessary with tissue from neonatal rats because P7 brain is not yet myelinated (Back and Rivkees 2004). Samples isolated either with or without Percoll gradients separated into similar populations in CD45 vs. FSC contour plots (data not shown) and two primary subpopulations, CD45low and CD45med. Between 1 and 3% of the population in each cell preparation was classified as CD45high. In all subsequent experiments cells were prepared without the use of Percoll gradients.

Compared to contralateral hemispheres, total CD45+ expression (before classifying into subpopulations) in injured hemispheres increased 3-fold ($p < 0.001$, $n = 7$). Thus, in contrast to peripheral leukocytes, the overall abundance of CD45+ cells increased significantly in the injured brain. A representative example of quadrant analysis of CD45/CD11b double-labeled cells in Fig. 2 shows that while in contralateral hemispheres, 80.9% of the cells were CD45low/CD11b- (Fig. 2a, lower left quadrant) and only 7.1% were CD45med-high/CD11b+ (Fig. 2a, top right quadrant), 60.3% of the cells acquired CD45med-high/CD11b+ expression (Fig. 2b, top right quadrant) by 24 h following injury.

We then used differential CD45 expression to determine whether the accumulated macrophages are resident or invading cells. A distinction between blood-borne macrophages and parenchymal cells of monocytic lineage (including quiescent or activated resident brain microglia) has been established in adult rodents based on differential CD45 expression levels: high CD45 expression levels in blood-born monocytes in contrast to low CD45 expression in quiescent microglial and a gradual increase in CD45 expression to medium-high levels during microglia activation (Carson *et al.* 1998; Campanella *et al.* 2002; Stevens *et al.* 2002). Contour plots delineated the presence of three peaks; CD45 low, med and high (not shown). Compared to contralateral hemispheres, where one major peak of CD45low (FI < 10^2) was observed (Fig. 2c), in injured tissue, a decrease in the CD45low peak, a clear rightward shift (up to and including FI = 10^3), and the resolution of two peaks, both CD45low and CD45med (10^2 < FI < 10^3) were detected 24 h post-reperfusion. Averaged data (Fig. 2d) indicated that cells from contralateral hemispheres expressed primarily CD45low (74 ± 15%) or CD45med (25 ± 15%) while CD45high macrophages represented less than 1% of the population. With injury, there was a 2-fold decrease in the abundance of cells expressing CD45low and a concomitant 2.2-fold increase in cells expressing CD45med (Fig. 2d), indicating a shift from quiescent to activated microglia. Following injury, the population of CD45high cells comprised less than 10% cells and varied from animal to animal (8.8 ± 6.7%).

We then colabeled CD45+ cells with a specific monocytic lineage marker, CD163, to confirm the lineage of cells in our experiments. CD163, originally identified as the hemoglobin scavenger receptor (Buechler *et al.* 2000), is constitutively expressed on tissue and perivascular macrophages and is up-regulated on microglia following various injurious conditions (Buechler *et al.* 2000; Roberts *et al.* 2004; Fabriek *et al.* 2005). CD163 expression was low on CD45low cells (Fig. 2e). Compared to contralateral hemispheres, in injured tissue there was an increase in the total number of CD45+/CD163+ double positive cells (49 ± 6.2% vs. 73 ± 8.4%, $p = 0.013$, $n = 5$) and a robust (10-fold) increase in CD163 expression within the CD45med population (FI > 10^2) (Fig. 2f). These data demonstrate not only increased CD45+/CD163+ cell numbers after injury, but also a higher cell surface expression of CD163 specifically in the population representing activated microglia.

Injury evolution is associated with further macrophage accumulation

At 48 h post-reperfusion, the distribution profile of CD45 was distinct from that at 24 h post-reperfusion (Fig. 3). The abundance of CD45high cells in injured tissue was substantially increased (reached approximately 30%, $p = 0.02$, $n = 4$, Fig. 3b). In addition, CD11b expression increased nearly 5-fold and quadrant analysis of contour plots (defined as for 24 h) from CD45/CD11b double-labeled cells indicated a shift in the CD45med/high/CD11b+ population from ~12% to 70% with injury (data not shown). In contralateral hemispheres, distribution among the three CD45 subpopulations was similar to that at 24 h (compare Fig 2d and Fig 3b). We did not observe accumulation of CD11b-/CD45low lymphocytes (Campanella *et al.* 2002), a finding consistent with our previous H&E findings (Derugin *et al.* 2005). In fact, this population decreased 24–48 h post-reperfusion.

Consistent with single labeled CD45 cells, the number of CD45/CD163 expressing cells increased in injured, but not in contralateral hemispheres, predominantly in CD45med activated microglia (not shown). Taken together these data demonstrate that beyond the acute injury phase accumulated macrophages are comprised of both activated microglia and increasing numbers of invading monocytes.

Peripheral and local cytokine levels in injured tissue increase rapidly and robustly after ischemia-reperfusion

Because leukocyte infiltration depends on a gradient of specific chemoattractant proteins, we next determined the effect of transient MCA occlusion on levels of 14 cytokines and chemokines in plasma, and injured and uninjured brain regions. Levels of cytokines that were within detectible levels are shown in Fig. 4. In plasma, the levels of cytokines IL-1β, IL-6 and the chemokine CINC-1 were significantly increased by 3 h MCA occlusion over control levels (2503 ± 1452 Vs. 241 ± 132 pg/mL for IL-1β, $p < 0.04$; 1998 ± 631 vs. 403 ± 265 pg/mL, $p < 0.03$ for IL-6; 4350 ± 2698 vs. 366 ± 209 pg/mL, $p < 0.015$ for CINC-1) (Fig. 4a). The levels of MCP-1 (625 ± 289 vs. 439 ± 148 pg/mL in control, $p > 0.05$) and IL-18 were not significantly changed at this time point. Upon reperfusion, systemic levels of IL-1β, IL-6, CINC-1, and MCP-1 increased further (Fig. 4a) and reached a maximum at 1 h post-reperfusion for IL-6 (2736 ± 1023 pg/mL, $p < 0.0001$ vs. control), CINC-1 (8721 ± 5720 pg/mL, $p < 0.0001$ vs. control) and MCP-1 (1104 ± 363 pg/mL, $p < 0.0001$ vs. control), and depending on the cytokine, returned to normal levels by 8–24 h post-reperfusion. IL-1β continued to increase over 4 and 8 h post-reperfusion (9384 ± 2610 and 7024 ± 2658 pg/mL, respectively, $p < 0.0001$ vs. control).

Compared to the response in the blood, the kinetics of the response in the tissue differed (Fig. 4b). IL-1β, IL-18, MCP-1 and CINC-1 in contralateral hemispheres remained unchanged over 24 h post-reperfusion compared to levels in naïve controls. Compared to contralateral tissue, in injured tissue, levels of IL-1β, MCP-1, and CINC-1 were significantly and transiently increased (Fig. 4b). Both local and systemic IL-1β peaked 4–8 h post-reperfusion. However, levels of chemokines MCP-1 and CINC-1 increased later in the brain than in plasma, beginning at 1–4 h after reperfusion, peaking when plasma levels of these chemokines were largely restored, 8 h after reperfusion. The levels of MCP-1 compared to corresponding contralateral levels (in pg/mg) were 8.9 ± 2.7 vs. 9.2 ± 2.8 (ns), 11.3 ± 5.9 vs. 6.3 ± 1.7 (ns), 41.1 ± 17.0 vs. 6.8 ± 2.0 ($p < 0.0005$), 59.3 ± 22.8 vs. 5.6 ± 3.4 ($p < 0.0001$), and 31.8 ± 15.3 vs. 7.8 ± 7.8 ($p < 0.01$) pg/mg protein at 0, 1, 4, 8, and 24 h post-reperfusion, respectively. These data demonstrate the existence of an ischemia-induced chemokine gradient governing leukocyte infiltration from blood to brain, including the major monocyte chemoattractant MCP-1.

MCP-1 expression is elevated in a subset of cells in the injured brain

We then used double-labeling immunofluorescence to determine whether the presence of elevated MCP-1 levels in injured brain was due to local production of MCP-1 in injured tissue. MCP-1 was weakly expressed in contralateral hemispheres (Figs 5a and b) anatomically matched to injured brain regions (Fig. 5c–f). Expression was evident primarily in the vasculature in uninjured hemispheres (Figs 5a and b) and in several cell types within injured tissue, including astrocytes (Figs 5c and e), macrophages (Figs 5d and f) and neurons (data not shown). While the majority of GFAP-immunoreactive astrocytes expressed MCP-1 in the injured region at 8 h post-reperfusion (Fig. 5c, arrows), this cell population was not seen in the core at 24 h (Fig. 5e, arrowheads). However, reactive astrocytes with thickened processes were abundant within the penumbra and a subpopulation of them expressed MCP-1 (Fig. 5e, arrow).

Discussion

Findings in this paper demonstrate for the first time that in neonatal focal stroke rapidly accumulated macrophages in ischemic-reperfused brain tissue are microglial cells rather than invading monocytes, a situation different from that reported in similar adult stroke models. Limited monocyte infiltration into acutely ischemic-reperfused neonatal brain does not result from lack of monocyte activation or lack of a monocyte chemoattractant gradient between the blood and injured brain regions, as CD45 and CD11b expression on circulating cells is increased and levels of multiple cytokines and leukocyte chemoattractant proteins, including MCP-1, are elevated in acutely injured neonatal brain.

Despite the traditional view that neonates have greater resistance to CNS injury compared to adults, it has become clear that immaturity in general as well as specific stages of brain development critically affect the susceptibility of the developing brain to hypoxic-ischemic insults and particular modes of neuronal death (reviewed in Vannucci and Hagberg 2004; Vexler and Ferriero 2006). The patterns of inflammation associated with brain injury are also age-dependent (reviewed in Hagberg and Mallard 2005; Vexler 2006). In a focal transient cerebral ischemia model in P7 rat (Derugin *et al.* 1998; Derugin *et al.* 2005), it is well documented that CD68-positive macrophages accumulate in injured tissue within hours after reperfusion (Derugin *et al.* 2000; Fox *et al.* 2005) and continue to increase over at least a one week period post-injury (Derugin *et al.* 2005; Dingman *et al.* 2006). However, the origin of these macrophages, whether invading monocytes or activated resident cells, is not known. This distinction is important for defining the relative contributions of systemic and local inflammatory responses to injury evolution.

Flow cytometry enables determination of cell surface marker expression on subpopulations of leukocytes based on differential expression levels of the common leukocyte antigen CD45; constitutive on peripheral leukocytes and low on resting microglia. Transient or a permanent MCA occlusion in adult animals results in both different extents and temporal patterns of CD45high/CD11b+ cell accumulation in the lesioned brain (Campanella *et al.* 2002; Stevens *et al.* 2002). In the present study, using a simplified and accelerated protocol for cell dissociation, we were able to discriminate between microglial cells (CD45low/CD45med) and infilltrated leukocytes (CD45high) within the brain. We found a progressive increase in CD45med cells at 24 and 48 h post-reperfusion, with a concomitant major decline in the number of CD45low cells in injured tissue. The number of CD45high cells remained below 10% at 24 h and reached approximately 30% by 48 h. Colabeling of CD45+ cells with CD163, an antigen selectively expressed on cells of monocyte lineage (Buechler *et al.* 2000; Roberts *et al.* 2004; Fabriek *et al.* 2005), confirmed the presence of activated microglia 24 h post-reperfusion and delineated the presence of a heterogeneous population of CD45high/CD163+ macrophages at 48 h, perhaps comprised of both activated microglia and invading macrophages.

Mechanisms that limit monocyte infiltration into acutely reperfused neonatal brain are yet to be understood. Leukocyte entry into the CNS can occur via multiple routes and is highly regulated under normal conditions (Ransohoff *et al.* 2003). Transmigration requires coordinated activation of leukocytes and endothelium, and the presence of appropriate chemoattractant gradients between the blood and brain. We found that expression of CD11b on peripheral leukocytes, an antigen required early in the transmigration process for ‘tethering’ and subsequent rolling along the endothelium, is significantly increased on peripheral leukocytes after ischemia-reperfusion. Other aspects of leukocyte activation during early post-natal period are poorly understood.

Maturation of the BBB (Saunders *et al.* 2000; Engelhardt 2003) can substantially affect leukocyte passage from blood to brain parenchyma. Despite the common belief that the BBB

in the neonate is more permissive than in adult, tight junctions are present even during embryonic development (Kniesel *et al.* 1996) and there are barrier mechanisms in the fetal brain not found in the adult (Saunders *et al.* 1999). By birth, the BBB is functional with no fenestrations (Engelhardt 2003). Age at time of insult can critically affect leukocyte transmigration, as was evident from a more preserved BBB in P0 and adult rats compared to P21 rats following inflammatory challenge (intrastriatum injections of IL-1β, TNFα or CINC-1) (Anthony *et al.* 1997; Anthony *et al.* 1998; Schnell *et al.* 1999; Blamire *et al.* 2000).

An insufficient gradient of chemoattractant molecules in the brain and/or a limited engagement of G-protein coupled chemokine receptors on monocytes with the appropriate ligands may also adversely affect leukocyte extravasation. MCP-1 and its receptor, CCR2 (Charo *et al.* 1994), play a central role in monocyte transmigration into the brain, as demonstrated by profound deficits in the recruitment of monocytes to sites of inflammation and injury in CCR2 deleted mice (Gosling *et al.* 1999; Gerard and Rollins 2001; Huang *et al.* 2001). A variety of cells have been shown to express MCP-1 after focal ischemia (Che *et al.* 2001) and trauma (Glabinski *et al.* 1996) in both adult and newborn rodent brain following hypoxia-ischemia (Xu *et al.* 2001) and excitotoxic insult (Galasso *et al.* 2000b). A pathophysiologic role of MCP-1 in neonatal brain injury was demonstrated by protection of the neonatal brain following functional inactivation of MCP-1 post-insult (Galasso *et al.* 2000b). Our data show that MCP-1 brain levels are profoundly elevated, up to 11 fold, and that MCP-1 is produced by several brain cell types, including endothelial cells and astrocytes.

Altered temporal-spatial expression of cytokines is an important modulator of trafficking of subsets of leukocytes after injury. In adult stroke, the most studied cytokines, IL-1β and TNFα, have been found to exacerbate brain damage by both inducing neuronal injury directly and via induction of glial and endothelial cells, with a consequent production of additional cytokines/chemokines and up-regulation of adhesion molecules (reviewed in (Allan *et al.* 2005; Lovering and Zhang 2005)). In adult rats, IL-1β injection preferentially triggers recruitment of neutrophils and propagates cytokine expression, whereas TNFα injection induces infiltration of monocytes, not neutrophils, and does not induce cytokine production (Blond *et al.* 2002). In P7 rats, increases in IL-1β were both substantial and sustained after MCA occlusion but were not associated with neutrophil extravasation or increased BBB permeability acutely after transient MCA occlusion (Dingman *et al.* 2004). Importantly, although substantial increases in local levels of IL-1β, MCP-1 and CINC-1 and lack of detectible levels of the anti-inflammatory cytokine IL-10 are consistent with a robust pro-inflammatory response, the specific lack of TNFα induction may contribute to limited/delayed monocyte infiltration in ischemia-reperfusion.

Biological functions of cytokines are not limited to regulation of leukocyte trafficking but affect a broad-range of processes after injury. In hypoxia-ischemia in neonatal rodents, for example, pharmacological inhibition of cytokines, either by IL-ra (Hagberg *et al.* 1996) or neutralizing anti-MCP-1 antibody (Galasso *et al.* 2000a), or deficiency of inflammatory cytokines, such as IL-18, IL-6, or of the FAS receptor (Hagberg *et al.* 1996; Hedtjarn *et al.* 2002; Graham *et al.* 2004), reduce injury and diminish microglial activation. In the immature brain, activated microglia/macrophages contribute to hypoxic-ischemic, ischemia-reperfusion and excitotoxic injury via several mechanisms (for a review see Vexler *et al.* 2006). The production of toxic products by microglia, such as inflammatory cytokines and chemokines (Szaflarski *et al.* 1995; Hagberg *et al.* 1996; Cowell *et al.* 2002; Hedtjarn *et al.* 2002; Cowell *et al.* 2003; Campbell *et al.* 2005) and high levels of NO (Tsuji *et al.* 2000), up-regulation of FAS receptor (Northington *et al.* 2001; Graham *et al.* 2004), complement receptor (Cowell *et al.* 2003; Ten *et al.* 2003, 2004) and iNOS (Dingman *et al.* 2006) can, in turn, further activate microglia, potentially propagating tissue injury. Furthermore, reduction of injury when macrophage

accumulation is diminished (Arvin *et al.* 2002; Dommergues *et al.* 2003) as opposed to unaffected injury severity when microglial activation is unchanged (Fox *et al.* 2005; Dingman *et al.* 2006) supports the notion that microglial activation is important in ischemic and excitotoxic injury in the immature brain. Taken together, our data on the relatively preserved BBB acutely after neonatal stroke (Dingman *et al.* 2004) and findings in this study that microglia cells rather than invading monocytes comprise the early macrophage population, and that local inflammation is profound suggest that the search for therapeutic agents designed to intervene with inflammatory processes early after neonatal stroke should be focused on molecules capable of crossing the BBB and accessing the brain parenchyma to target microglial cells.

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Abbreviations used

BBB, blood–brain barrier
CINC′ 1, cytokine-induced neutrophil chemoattractant protein-1
CNS, central nervous system
DW, diffusion-weighted
IL-1b, interlukin 1b
FI, fluorescence intensity
MCA, occlusion, middle cerebral artery occlusion
MCP-1, monocyte chemoattractant protein-1
P7, post-anatal day 7
PBS, phosphate-buffered saline
TNF-α, tumor necrosis factor

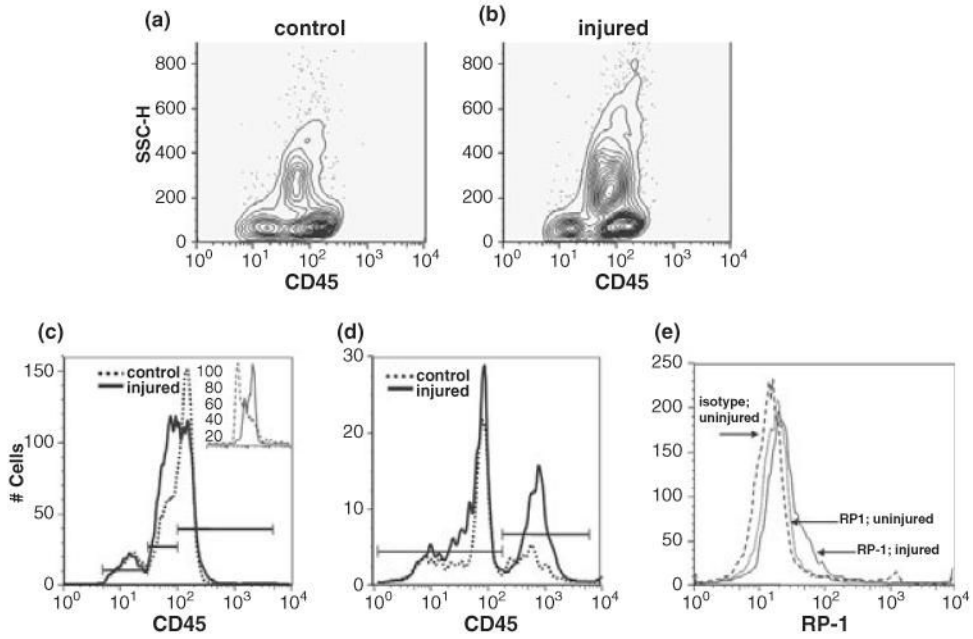

Fig. 1.

Peripheral leukocytes are activated 24 h after MCA occlusion in P7 rats. (a and b) Three populations of CD45+ cells are detected in CD45 vs. SSC contour plots. (c and d) Increases in CD45med (c) and CD11b (d) expression are detected with injury. In uninjured animals the percentage of CD45+ cells classified as low, med, or high (bars) is, respectively, 14 ± 7.7%, 36 ± 7.8%, and 50 ± 7.1% and in injured animals, these ratios shift to 10 ± 7%, 46 ± 4% and 44 ± 3.7. Statistically significant shifts as a result of injury occur in the CD45medium/CD11b + positive populations ($p = 0.05$, $n = 4$). Histograms in this and all subsequent figures show CD45 gated cells. Bars in CD11b histograms represent negative and positive subpopulations. Inset in (c) shows isotype control antibody (dashed line) compared to CD45 antibody labeling of control (solid) samples. (e) MCA occlusion results in increased RP-1 expression. Quantification of RP-1 positive cells to the right of the isotype matched control antibody (dashed curve) indicates injury results in an increase in RP-1+ cells from 18 ± 9.7% to 22 ± 4.7% (solid curve, control; dotted, injured) of the CD45 population ($p > 0.5$, $n = 3$). Shown are data from a representative experiment.

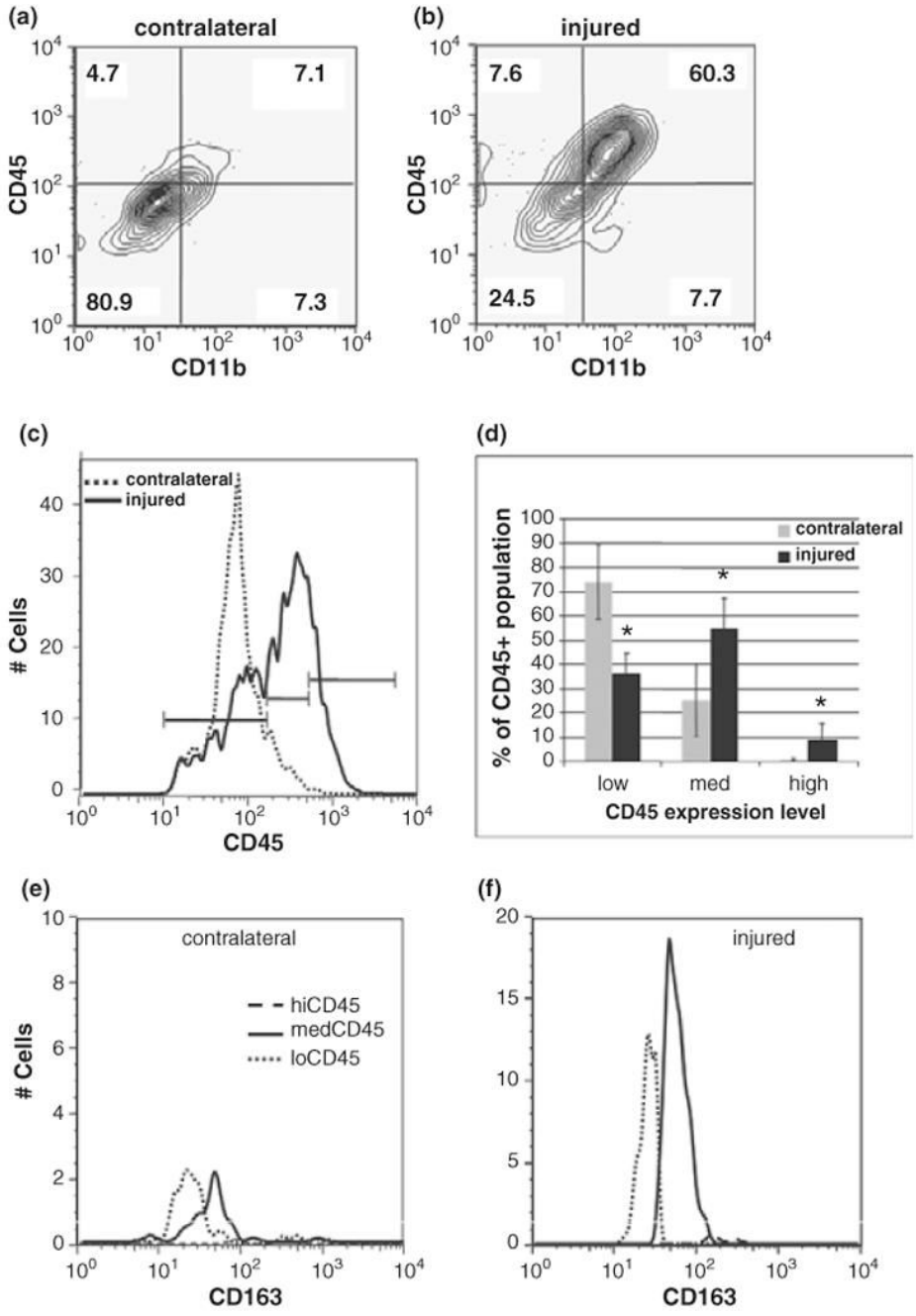

Fig. 2.

Macrophages in the brain are predominantly activated microglia, not invading monocytes, 24 h post-ischemia reperfusion. (a and b) The number of activated leukocytes (CD45+/CD11+) increases from 7.1% in uninjured (a) to 60.3% in injured tissue (b). Values represent the percent of CD45 gated cells present in that quadrant. Note that in quadrant analysis, CD45+ cells contain both CD45med and CD45high populations. Data are representative of 4 independent experiments. (c) CD45 histograms indicate a shift towards higher intensity and a broadening of the curve with injury. Bars indicate the boundaries of low, medium and high CD45 expression levels. Dotted = contralateral, solid = injured. (d) With injury, there is a shift in the CD45 population from CD45low (36 ± 7.9%) to CD45med (55 ± 12.3%). Less than 10% (8.8

± 6.7%) of the population in injured tissue is CD45high. Data are expressed as mean ± SD, $n = 11$. *$p < 0.005$ compared to contralateral. Grey bars = contralateral, solid = injured. (e and f) Expression of monocytic lineage marker CD163 is rapidly increased on activated microglia 24 h post-reperfusion. In contralateral hemispheres, CD163 expression is minimal (e) whereas in injured tissue CD163 expression is induced in CD45med cells (f). Dotted line, CD45low; solid line, CD45med, dashed line, CD45high. Shown are data from a representative experiment, $n = 5$.

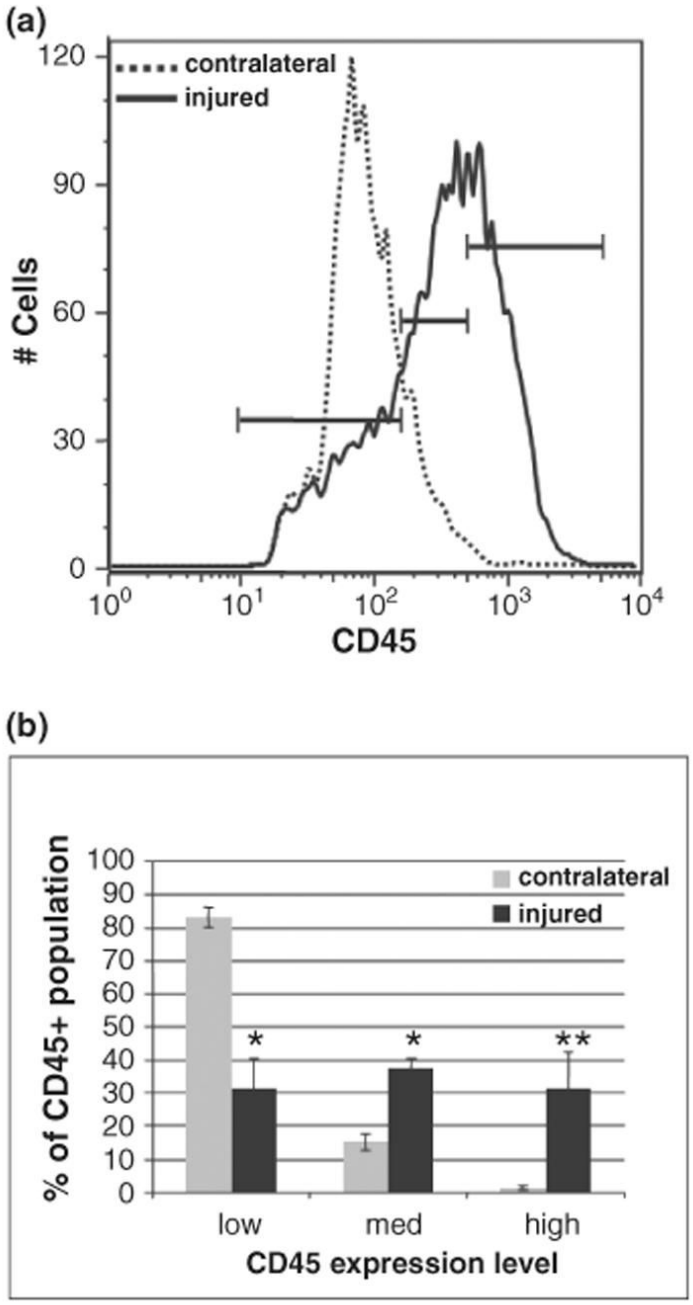

Fig. 3.

Macrophages continue to accumulate at 48 h post-reperfusion. (a) CD45 histograms show a rightward curve shift with injury evolution. (b) CD45low cells continue to predominate in contralateral hemispheres (85 ± 3.9%, 14 ± 3.4%, 1.2 ± 0.6%, respectively, for low, med and high expression; $n = 5$), whereas with injury the distribution among the three subpopulations changes dramatically (31 ± 7.3%, 39 ± 3.0% and 30 ± 9%, respectively, for low, med and high; $n = 4$). Mean ± SD. *$p < 0.005$ for low, med; **$p < 0.02$ for high populations compared to contralateral.

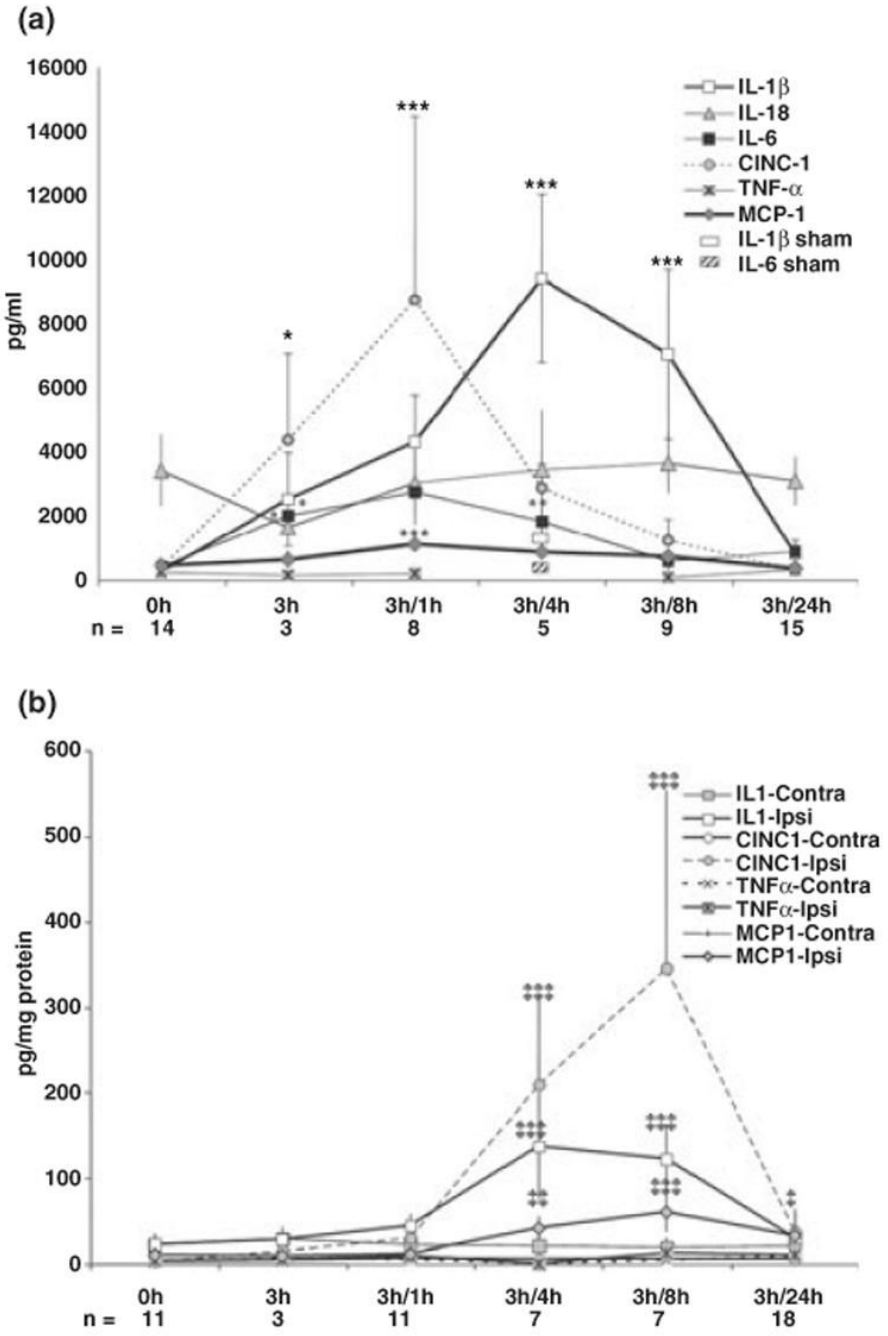

Fig. 4.

Cytokine and chemokine levels are increased in plasma and injured brain tissue but not in contralateral hemisphere within 24 h after reperfusion. A significant transient increase in IL-1β, IL-6, CINC-1, and MCP-1 occurs first in plasma (a, 0–8 h post-reperfusion) and then in the injured brain (b, 4–8 h post-reperfusion). Measurements were performed using the multiplex cytokine kit (LINCO Research) in animals identified by DW-MRI as injured. Data are expressed as mean ± SD, n = 3–17 per time point, as indicated for individual time points. In a: *$p < 0.04$ vs. control; **$p < 0.015$ vs. control; ***$p < 0.0001$ vs. control. In b: §$p < 0.04$; §§$p < 0.01$; §§§$p < 0.001$ vs. value in the corresponding contralateral hemisphere.

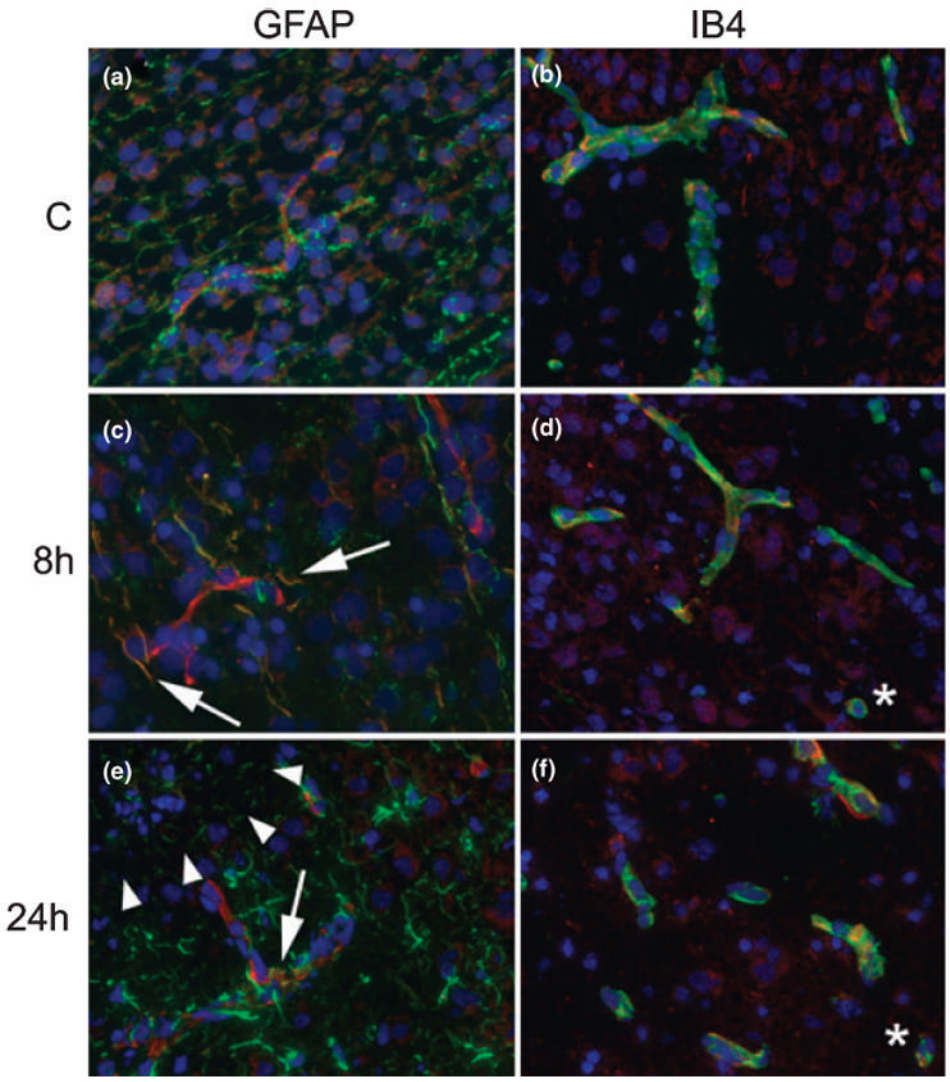

Fig. 5.

MCP-1 expression is elevated in multiple cell types within injured regions. (a and b) MCP-1 expression is evident in the vasculature in contralateral hemisphere. (c and d) At 8 h post-reperfusion, MCP-1 is expressed in astrocyte processes (c), vessels and macrophages (d) in injured regions. (e and f) At 24 h post-reperfusion, continued expression is detected in the vasculature, astrocyte processes in the penumbra, and macrophages. Arrows in c and e indicate coexpression of MCP-1 with GFAP in astrocyte processes; asterisks in d and f indicate coexpression in cells that likely represent microglia. Arrowheads in e indicate the boundary between injury core and penumbra. All images were captured at 20 ×. In all images red = MCP-1 and blue = nuclei (DAPI), green = GFAP (a, c, e) or IB4 (b, d, f).